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# Epstein-Barr Virus Recombinants with Specifically Mutated BCRF1 Genes

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**Epstein-Barr virus (EBV) recombinants with specifically mutated BCRF1 genes were constructed and compared with wild-type BCRF1 recombinants derived in parallel for the ability to initiate and maintain latent infection and growth transformation in primary human B lymphocytes. A stop codon insertion after codon 116 of the 170-codon BCRF1 open reading frame or deletion of the entire gene had no effect on latent infection, B-lymphocyte proliferation into long-term lymphoblastoid cell lines (LCLs), or virus replication. LCLs infected with the stop codon recombinant were indistinguishable from wild-type recombinant-infected LCLs in tumorigenicity in SCID mice. However, mutant BCRF1 recombinant-infected cells differed from wild-type recombinant-infected cells in their inability to block gamma interferon release in cultures of permissively infected LCLs incubated with autologous human peripheral blood mononuclear cells. This is the first functional assay for BCRF1 expression from the EBV genome. BCRF1 probably plays a key role in modulating the specific and nonspecific host responses to EBV infection.**

Epstein-Barr virus (EBV) is a ubiquitous B-lymphotropic herpesvirus which causes infectious mononucleosis and lymphoproliferative disease in immunosuppressed humans (12). The virus is usually transmitted between humans in saliva, infects oropharyngeal epithelium, and then spreads to circulating B lymphocytes (24). As with other viruses, host resistance to EBV infection is first mediated by interferon (IFN) and natural killer (NK) cells and later by antibody and virus-specific, major histocompatibility complex-restricted cytotoxic T cells (16, 24, 30, 41). After primary EBV infection, latently infected B lymphocytes persist in small numbers. The latently infected B lymphocytes are capable of expressing a set of six nuclear proteins (EBNAs), two integral latent membrane proteins (LMPs), and two small RNAs (EBERs) (16). Antibodies to the EBNAs and cytotoxic $CD8^+$ T lymphocytes specific for EBNAs (except EBNA1) or LMPs are maintained for life (30), indicating that latently infected cells continue to provide these antigenic stimuli to the immune system. Thus, in establishing persistent latent infection in B lymphocytes and in continuing to express proteins which are recognized by cytotoxic T lymphocytes, EBV must establish a balanced and regulated relationship with many elements of the normal immune system.

Because of the importance of the complex interaction between EBV and the immune system in human infection, this study focuses on BCRF1, an EBV gene which was recently discovered to be 84% homologous to the human interleukin 10 (IL-10) gene (25). BCRF1 is likely to have an important unique role in EBV infection, since EBV is the only human herpesvirus to encode an IL-10 homolog (3, 25). Not surprisingly, given the extensive homology to IL-10, BCRF1 has many of the known in vitro effects of IL-10 (36). Murine IL-10 is produced by a subset of T lymphocytes that can help B lymphocytes to proliferate or differentiate (8, 9, 28). Murine IL-10 inhibits release of cytokines, including IFN-γ, from $T_H1$ lymphocytes, which preferentially mediate delayed-type hypersensitivity. Human IL-10 can act on macrophages to down modulate IFN-γ and tumor necrosis factor alpha secretion by human peripheral blood mononuclear cells (PBMCs) (14). Since BCRF1 is expressed late in the EBV lytic cycle (15, 37), BCRF1 could function in primary or reactivated EBV infection to blunt IFN synthesis.

BCRF1 could also have some direct effects on B-cell growth. IL-10 enhances the viability of resting B lymphocytes and supports the growth and differentiation of activated B lymphocytes (10, 33). Since IL-10 can also enhance B-lymphocyte proliferation in conjunction with IL-4 (33), IL-10 or BCRF1 could be important in the initiation of latent B-lymphocyte infection and cell growth transformation or in stimulating an aspect of cell growth necessary for virus replication.

To further investigate the role of BCRF1 in initiation or maintenance of latent lymphocyte infection, growth transformation, or lytic virus infection in B lymphocytes, we have constructed EBV recombinants with specifically mutated BCRF1 genes. The recombinants were compared with wild-type (WT) virus for the ability to infect, growth transform, and replicate in B lymphocytes in vitro. Comparison of the interaction of cells permissively infected with BCRF1 mutant or WT EBV recombinants with human PBMCs enabled us to identify for the first time an effect of BCRF1 secreted from lytically infected B lymphocytes on IFN synthesis by human PBMCs.

## MATERIALS AND METHODS

**Cell lines, cosmids, and plasmids.** P3HR-1 clone 16 (29) cells were obtained from G. Miller, Yale University, and maintained in complete medium, which consists of RPMI 1640 supplemented with 10% fetal calf serum. Plasmid pSVNaeZ and cosmids SalA and pDVcosA2 have been described previously (39). Cosmids were packaged with the Gigapack packaging extract and used to infect *Escherichia coli* PLK-A (Stratagene). An oligonucleotide consisting of CTAGTCTA-

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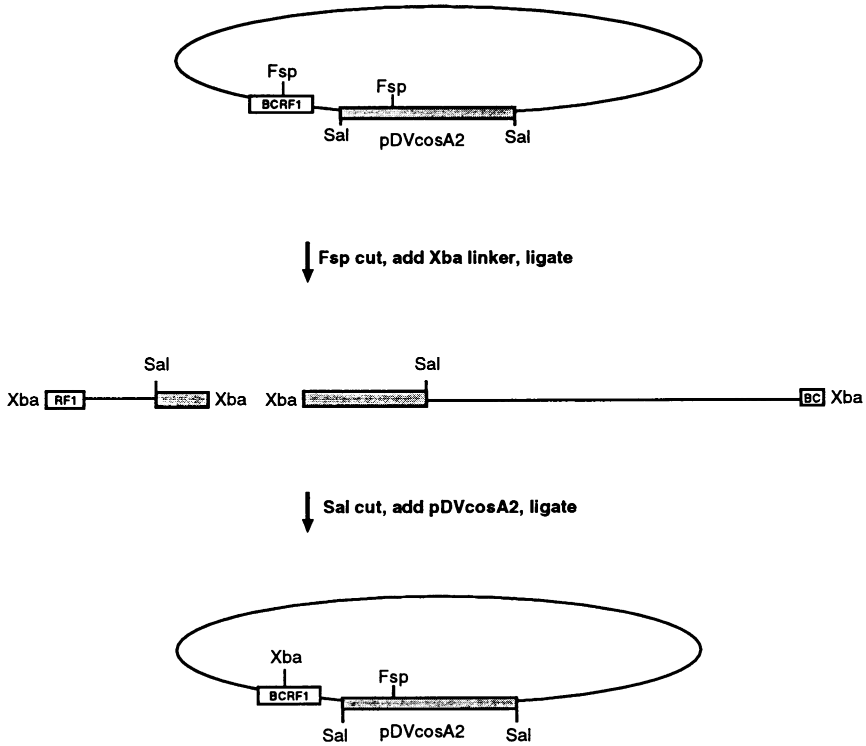


FIG. 1. The SalA cosmid was digested with *Fsp*I, ligated with an excess of *Xba*I linker, and digested with *Sal*I. The resulting fragments were heated to 65°C to remove the unligated strand of the *Xba* linker and gel purified. The *Xba*-*Sal* EBV DNA fragments were gel purified and treated with T4 kinase to phosphorylate the 5′ termini of the *Xba* linker. The phosphorylated fragments were ligated with *Sal*I-cut cosmid vector pDVcosA2 to regenerate the complete SalA cosmid with the *Xba* linker inserted in the *Fsp* site.

GACTAG, which encodes nonsense in all three reading frames and includes an *Xba*I site (New England Biolabs), was inserted into the BCRF1 open reading frame (ORF) at a unique *Fsp*I site in the EBV SalA fragment (3) (Fig. 1). A deletion of the BCRF1 ORF was constructed by cleavage of the *Xba*I linker-converted SalA cosmid with *Xba*I, BAL 31 digestion, and religation.

**PCR analysis, Southern blotting, DNA sequencing, immunoblotting, and ELISA.** Oligonucleotides corresponding to EBV nucleotides 99009 to 99029 and 10120 to 10140 (3) were synthesized by using an Applied Biosystems synthesizer and purified (39). DNA for polymerase chain reaction (PCR) analysis was prepared from $10^6$ cells by boiling, proteinase K treatment, and boiling (39). RNA was purified from $10^7$ cells with RNAzol (5), treated with RNase-free DNase (RQ1; Promega) for 30 min, phenol extracted, and reverse transcribed with avian myeloblastosis virus reverse transcriptase (Promega) at 42°C for 1 h. The resultant cDNA was amplified by using *Taq* polymerase (Perkin-Elmer Cetus) as described previously (39). The procedures used for Southern blotting, DNA sequencing, immunoblotting, and enzyme-linked immunosorbent assay (ELISA) for IFN have been described elsewhere (6, 25, 43).

**Transfection and induction of lytic virus infection, virus and primary B-lymphocyte preparations, and infection.** SalA cosmid DNA was cleaved with *Sal*I to release the EBV DNA. The cleaved DNA (20 μg) was mixed with pSVNaeZ (40 μg) and electroporated into $5 \times 10^6$ P3HR-1 cells with a Bio-Rad Gene Pulser (39). Two or three days later, the transfected cells were lysed by three cycles of freezing and thawing in the medium and passed through a 0.45-μm-pore-size filter. The filtrate was centrifuged at $8{,}000 \times g$ for 2 h, and the resultant pellet was resuspended in 1.0 ml of complete medium and used to infect $5 \times 10^7$ PBMCs from an EBV-seronegative donor (39). The infected cells were plated into 320 microwells and fed weekly with complete medium. PBMCs were isolated on a Ficoll-Hypaque gradient as previously described (38).

For passage of virus from latently infected lymphoblastoid cell lines (LCLs), lytic virus infection was induced by the addition of 20 ng of 12-tetradecanoyl phorbol acetate (TPA) per ml to the growth medium. Cells were harvested for immunoblot analysis 72 h after TPA treatment. TPA-treated cells ($5 \times 10^5$) were lethally irradiated with 90 Gy and mixed with $15 \times 10^6$ PBMCs from an EBV-seronegative donor at a ratio of $5 \times 10^3$ LCLs and $1.5 \times 10^5$ PBMCs in 150 μl per microwell. Transformed LCLs were obtained 3 to 6 weeks after cocultivation.

**IFN assays.** LCLs were treated with 20 ng of TPA per ml to induce lytic EBV infection. Seventy-two hours later, the cells were lethally irradiated with 90 Gy and washed twice in complete medium, and $10^6$ cells were incubated for 5 days with $5 \times 10^6$ PBMCs in 1 ml of complete medium. The supernatant medium was assayed for IFN-γ by ELISA after 5 days (42). Mean values for IFN concentration were compared by Student's unpaired *t* test. The number of transforming virus particles released from each LCL used in the IFN assays was measured in a long-term immortalization assay. An aliquot of the induced LCLs containing $5 \times 10^5$ cells was mixed with $15 \times 10^6$ PBMC and plated at a ratio of $5 \times 10^3$ LCLs and $1.5 \times 10^5$ PBMCs in 150 μl per microwell. Newly transformed colonies of B lymphocytes were counted 4 to 6 weeks later.

**Tumor induction in SCID mice.** SCID mice were raised in a pathogen-free breeding colony at the University of Massachusetts. Immunoglobulin levels were measured to exclude residual immune function in the SCID mice prior to injection of LCLs. A total of $8 \times 10^5$ or $4 \times 10^6$ cells from each LCL were washed in phosphate-buffered saline and injected intraperitoneally into 4- to 6-week-old SCID mice. Mice were inspected daily and sacrificed if moribund. All surviving animals were sacrificed after a minimum of 104 days.

## RESULTS

**EBV recombinants with a WT or an S mutant BCRF1 ORF.** The strategy for derivation of EBV recombinants with a WT or a nonsense codon (S mutant) BCRF1 ORF was that of linked transformation marker rescue from P3HR-1 cells (39). P3HR-1 cells are latently infected with a lytic replication-competent EBV strain which has undergone deletion of a DNA fragment which includes part of the EBNA-LP ORF and all of the EBNA2 ORF (29). As a consequence of this deletion, EBV from P3HR-1 cells cannot transform primary B lymphocytes (29, 35). However, transfection of P3HR-1 cells with a cloned WT EBV DNA fragment which includes the DNA deleted from P3HR-1 and induction of lytic virus infection results in a low frequency (approximately 1 in $10^5$) of homologous recombination, incorporation of WT EBV DNA at the site of the P3HR-1 deletion, and marker rescue of the transformation phenotype (6, 11). When the progeny virus is used to infect $5 \times 10^7$ PBMCs from an EBV-seronegative donor and the infected cells are plated into 320 microwells, LCLs grow only in those wells containing a recombinant virus-infected B lymphocyte (6, 39). Using a cosmid-cloned EBV DNA fragment which spans the deletion and includes the EBER genes, we transferred a deletion of the EBER genes from the cloned EBV DNA into 20% of the recombinant virus genomes, even though the EBERs are over 30 kb away from the marker rescue site (39). Since the BCRF1 ORF is 3 kb closer to the marker rescue site, a mutation in the BCRF1 ORF is expected to be incorporated into at least 20% of the transforming, marker-rescued EBV recombinants, assuming that the mutation does not adversely affect the ability of the recombinant to initiate or maintain B-lymphocyte growth transformation.

EBV recombinants with a WT or mutant BCRF1 ORF were generated by transfecting P3HR-1 cells with a cosmid-cloned EBV DNA fragment that was competent for marker rescue of transformation and in which the BCRF1 ORF had been mutated. A 14-bp oligonucleotide containing an *Xba*I site and encoding nonsense in all three reading frames was inserted into codon 116 of the 170-codon BCRF1 ORF (Fig. 1). The insertion site of the mutated cosmid was first confirmed to be as expected by DNA sequencing across the oligonucleotide insertion site. When the resultant virus was used to infect PBMCs and the infected cells were plated into 320 microwells, 60 presumably clonally derived cell lines resulted. These were screened by PCR using oligonucleotide primers which should yield a 231-bp amplified segment from a WT BCRF1 ORF and a 245-bp amplified segment from the S mutant BCRF1. As expected from the previous finding that only 20% of the transforming recombinants from a transfection with an EBER deletion cosmid had the EBER deletion and 80% had the WT P3HR-1 EBER gene, 43 of the 60 clones (72%) had only the WT BCRF1 from the P3HR-1 genome (data not shown). The PCR product was 231 bp and was resistant to *Xba*I cleavage. Fourteen of sixty clones (23%) had only the S mutant BCRF1 and gave a product of 245 bp, which was cleaved by *Xba*I (data not shown). The remaining three clones were coinfected with an S mutant EBV recombinant and with nonrecombinant P3HR-1 EBV and had a 231-bp band, a 245-bp band, and a third band consisting of a hybrid between the two (data not shown).

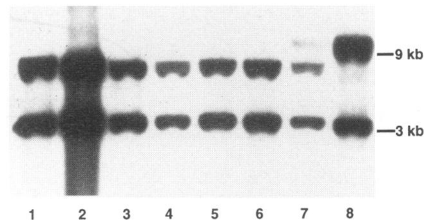


FIG. 2. DNA from LCLs transformed by EBV recombinants was analyzed by Southern blotting. DNA was cleaved by *Bam*HI and *Xba*I and probed with the *Bam* C fragment of EBV. WT virus DNA contains a 9-kb *Bam*C and a 3-kb *Bam* W fragment (both fragments hybridize to the *Bam*-C probe because of the presence of repetitive sequences common to *Bam*-C and *Bam*-W). S mutant *Bam* C is cleaved into 6- and 3-kb fragments by *Xba*I, the latter comigrating with *Bam*-W. Lanes: 1 to 6, DNA from cell lines infected by S mutant BCRF1 EBV recombinants; 7, DNA from a cell line coinfected with an S mutant BCRF1 EBV recombinant and parental P3HR-1 which has WT BCRF1; 8, DNA from a cell line infected with a WT BCRF1 EBV recombinant.

Southern blot analysis of DNA from representative clones cut with *Bam*HI and *Xba*I confirmed the PCR results (Fig. 2). DNA from an LCL infected with WT virus (lane 8) contains a 9-kbp *Bam* C fragment which is unaffected by *Xba*I digestion and a 3-kbp *Bam* W fragment, both of which hybridize to the *Bam*-C probe. The 3-kbp-long internal repeat in EBV DNA begins in *Bam*-C, and the *Bam* site between C and W is the first *Bam* site in each copy of the repeat. Since there are multiple copies of *Bam*-W, the *Bam*-C probe hybridizes almost as intensely to *Bam*-W as to itself. Lane 1 to 6 in Fig. 2 all contain LCLs which by PCR were singly infected with an S mutant BCRF1 EBV recombinant. All six lack the 9-kbp WT BCRF1 *Bam*-C, as *Xba*I cleaves the mutant *Bam*-C DNA to 6- and 3-kb fragments. DNA from an LCL which is coinfected with an S mutant BCRF1 EBV recombinant and with nonrecombinant P3HR-1 is shown in lane 7. *Xba*I cleaves most of the *Bam*-C DNA to 6 kbp, indicating that there is more S mutant than WT BCRF1 DNA in this LCL. To establish the sensitivity with which WT BCRF1 would have been detected in DNA from those LCLs which appear to lack BCRF1 by Southern blotting, serial dilutions of WT BCRF1 recombinant-infected LCL DNA were analyzed in control lanes; 1:10 and 1:100 dilutions of WT BCRF1 LCL DNA were detectable (data not shown). These data indicated that the S mutation in BCRF1 does not interfere with the ability of S mutant BCRF1 EBV recombinants to maintain latent B-lymphocyte infection or growth transformation in the absence of WT BCRF1.

**Expression of S mutant or WT BCRF1 RNA.** BCRF1 protein expression in cells which are highly permissive for EBV replication (24) has been detected by using a polyclonal rabbit serum generated against a BCRF1 fusion protein expressed in *E. coli* (37). BCRF1 protein expression has not been demonstrated in EBV-infected cells less permissive for EBV replication or with use of monoclonal antibodies. To confirm that S mutant BCRF1 RNAs were expressed in LCLs infected with mutant recombinant viruses, RNA was prepared from cells treated with TPA, cDNA was made from the RNA and was amplified by PCR using the oligonucleotide primers flanking the mutation site, and the product was analyzed on gels before and after cleavage with *Xba*I. A PCR product of the expected size was obtained from S mutant recombinant-infected LCL

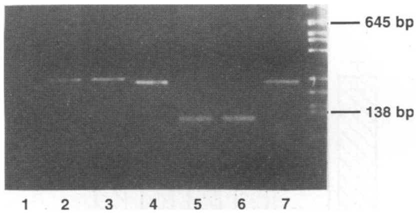


FIG. 3. PCR of cDNA from RNA of an LCL infected with S mutant BCRF1 EBV recombinant. The LCL was treated with TPA to increase lytic protein expression, and RNA was isolated. The RNA was treated with DNase, reverse transcribed, and subjected to PCR analysis. Lanes: 1, RNA without reverse transcriptase; 2, BCRF1 S mutant LCL cDNA; 3, BCRF1 S mutant cosmid DNA control; 4, BCRF1 WT cosmid DNA control; 5, BCRF1 S mutant LCL cDNA, cut with *Xba*I; 6, BCRF1 S mutant cosmid DNA, cut with *Xba*I; 7, BCRF1 WT cosmid DNA, incubated with *Xba*I.

cDNA and was cleavable with *Xba*I (Fig. 3). The PCR was detecting only cDNA derived from RNA, since no product was obtained in parallel controls done without addition of reverse transcriptase (Fig. 3).

**Comparison of the abilities of S mutant and WT BCRF1 EBV recombinants to initiate latent infection, growth transform primary B lymphocytes, form tumors in SCID mice, and initiate lytic virus infection in vitro.** LCLs infected with S mutant or WT BCRF1 EBV recombinants were also compared by immunoblotting with polyclonal EBV immune human sera for their spontaneous permissivity for EBV lytic infection. Both types of LCLs varied in permissivity over similar ranges. Immunoblots of representative LCLs are shown in Fig. 4.

To compare the ability of S mutant and WT BCRF1 EBV recombinants to initiate latent infection and to growth transform primary B lymphocytes, virus was passaged to primary B lymphocytes from four S mutant or four WT recombinant-infected LCLs which lacked coinfecting P3HR-1 genomes. Spontaneously permissive LCLs were further induced to lytic virus infection, lethally irradiated, and cocultivated with primary B lymphocytes. Macroscopic growth of newly arising LCLs infected with either S mutant or WT EBV recombinants was evident at between 4 and 6 weeks after plating. Similar fractions of the wells were positive. The microscopic morphology of the cells and the extent of cell growth in clumps or independent of clumps were similar for S mutant and WT recombinant-infected cells. After 8 weeks in culture, the doubling times of the S mutant- and WT-infected LCLs were compared. The two doubling times varied similarly, with LCLs doubling between 48 and 72 h, irrespective of the BCRF1 genotype of the recombinant virus.

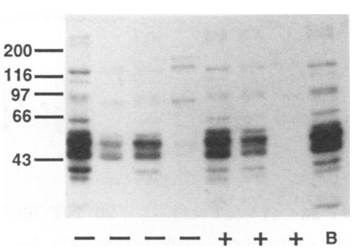


FIG. 4. Levels of spontaneous virus replication were compared by immunoblotting lysates from cells infected with WT BCRF1 EBV recombinants (+) or S mutant BCRF1 EBV recombinants (−), using human sera reactive against early replicative antigens. B, B95-8 lysate. Molecular sizes are shown at the left in kilodaltons.

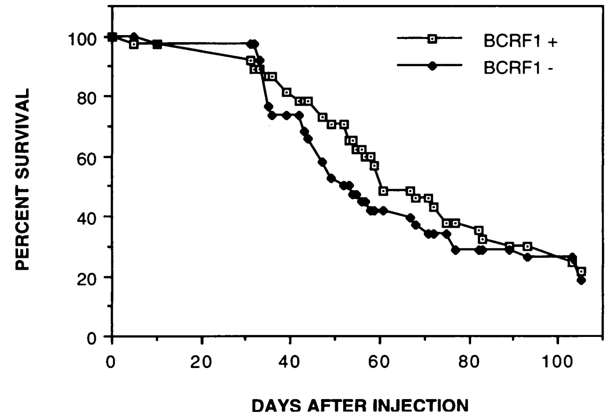


FIG. 5. Ten mice were injected intraperitoneally with $4 \times 10^6$ cells for each cell line used. Pooled data from four S mutant BCRF1 and four WT BCRF1 cell lines are shown.

To evaluate whether BCRF1 expression in a small fraction of cells permissive for virus replication could have a B-cell growth factor effect on neighboring latently infected cells in vivo, the growth of spontaneously permissive LCLs infected with either specifically mutated or WT BCRF1 recombinants was measured in SCID mice. In SCID mice, EBV-infected cells can grow into solid tumors and invade tissues (26, 34). Since BCRF1 is expressed in late lytic infection, an effect on cell growth or invasiveness of the BCRF1 secreted from cells permissive for virus replication might be evident in SCID mice. Four representative LCLs infected with S mutant BCRF1 EBV recombinants and four LCLs infected with WT BCRF1 recombinants were used in SCID mouse experiments. The LCLs infected with WT or mutant BCRF1 recombinants were similar in the extent of spontaneous lytic protein expression in vitro as measured by immunoblotting (not shown). Each LCL was injected intraperitoneally at two doses ($8 \times 10^5$ and $4 \times 10^6$ cells). Ten SCID mice were injected with each dose. The mice were inspected daily and sacrificed when moribund. Autopsy was performed on mice spontaneously dying of tumor or after sacrifice. The experiments were terminated after 104 days, and autopsies were performed on the remaining mice to determine whether they were tumor free. Tumorigenicity and lethality of the S mutant and WT BCRF1-containing LCLs were similar. Following injection with $4 \times 10^6$ of either type of LCL, tumors were similarly distributed in abdominal viscera, thymus, and splenic tissues. Eighty percent of the animals died from tumor progression, with mean times to death of 59 and 58 days for S mutant- and WT-infected LCLs, respectively (Fig. 5). Following the injection of $8 \times 10^5$ cells, the mean time to death was 61 and 83 days for S mutant- and WT-infected LCLs, respectively, a difference which was not statistically significant (data not shown).

**WT BCRF1 inhibits IFN synthesis by PBMCs.** To search for an assay which could detect an effect of WT BCRF1 secreted from infected LCLs, we focused on IFN-γ secretion by T and NK cells, in response to EBV infection of PBMCs, in vitro (2, 21, 40). IL-10 and BCRF1 from transfected Cos cells can act on macrophages to inhibit T and NK synthesis of IFN-γ and tumor necrosis factor alpha (9, 13, 42). Thus, the IFN-γ-inhibiting effects of BCRF1 secreted from lytically infected cells could counteract the IFN-γ-inducing effects of virus released from such cells. LCLs were exposed to TPA to induce additional permissivity for lytic EBV infection and were mixed with PBMCs so that they could interact in vitro. Since the time

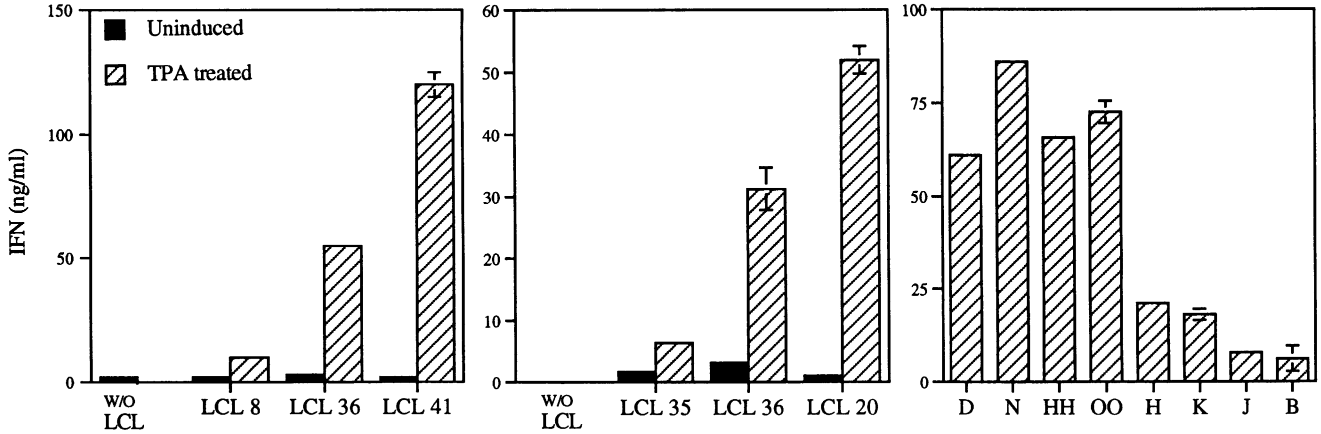


FIG. 6. IFN-γ synthesis in response to infection of PBMCs by EBV released from S mutant BCRF1 EBV or WT BCRF1 EBV-infected LCL was measured by ELISA. Standard error of the mean is shown by error bars when greater than 2 ng/ml. LCLs 20, 36, 41, D, N, HH, and OO are infected with S mutant EBV; LCLs 8, 35, H, K, J, and B are infected with WT BCRF1 EBV recombinants. W/O LCL, control PBMCs without addition of EBV-infected cells.

of maximum late lytic cycle EBV expression occurs 72 h after TPA treatment, 72 h was allowed to elapse between exposure to TPA and washing, irradiation to prevent further LCL growth, and mixture with PBMCs. The concentration of LCLs, the concentration of PBMCs, and the ratio of LCLs to PBMCs were higher than in typical virus passage experiments so as to maximize the amount of virus and BCRF1 released from the LCLs, the concentration of IFN released from PBMCs, and the effect of BCRF1 on PBMCs. If WT BCRF1 acted to reduce IFN-γ synthesis in such a system, S mutant BCRF1 might lack such an effect, resulting in higher-level IFN-γ secretion.

The first result from this series of experiments was that rapid proliferation and acidification of the medium occurred, indicating substantial metabolic activity, probably due to EBV-driven B-lymphocyte proliferation and T and NK cell activation. Only LCLs which had been rendered more permissive for virus replication stimulated such metabolic activity. IFN-γ was readily detected in many cultures at 5 days after mixture of LCLs and PBMCs. The extent of induction of permissive infection in the LCLs correlated directly with the amount of IFN released (see below). PBMCs from individual donors varied in the extent of IFN secretion, but individual donors were relatively consistent in their IFN-γ response. The IFN-γ response was not dependent on the presence of antibody to EBV in the donor serum.

One seronegative donor, whose PBMCs gave consistent IFN-γ release, was used in three separate experiments shown in Fig. 6 and 7 and Table 1. Six WT BCRF1 recombinant EBV-infected LCLs (8, 35, H, K, J, and B) were compared with seven S mutant recombinant EBV-infected LCLs (36, 41, 20, D, N, HH, and OO). While the absolute levels of IFN varied among the three experiments shown in Fig. 6, in all instances, TPA-treated, S mutant BCRF1 recombinant-infected LCLs stimulated the release of at least threefold higher levels of IFN than did WT BCRF1-infected LCLs. Mean concentrations of IFN released in response to mutant BCRF1 versus WT BCRF1 LCLs were 68 and 11.6 ng/ml, respectively ($P < 0.001$). TPA-treated LCLs induced higher-level IFN responses than did non-TPA-treated LCLs, consistent with virus replication being an important effector of IFN induction. Carryover of TPA was not responsible for IFN secretion, since TPA-treated, washed, EBV-negative lymphoblasts cocultivated with PBMCs did not induce IFN secretion over background (data not shown). These results are the first demonstration of an activity for BCRF1 secreted from permissively infected cells and prove that S mutant BCRF1 is functionally less active or inactive in blocking IFN synthesis.

To confirm that the amounts of EBV replication in the TPA-induced, S mutant and WT BCRF1-infected LCLs used in these experiments were similar, immunoblotting for EBV lytic proteins was performed on lysates of the LCLs used in the first two cocultivation experiments. S mutant and WT-infected LCLs exhibited similar induction of lytic proteins with TPA treatment (Fig. 7). In addition, the numbers of permissively infected cells in each of the LCL cultures used in IFN release experiments were compared by cocultivating dilutions of the LCLs with primary B lymphocytes in a long-term immortalization assay. Endpoint dilutions of aliquots of each TPA-treated, irradiated S mutant or WT BCRF1-infected LCL were mixed with PBMCs and plated in microwells as described in Materials and Methods. Individual colonies of B lymphocytes transformed de novo were counted 4 weeks later. The numbers of permissively infected cells in TPA-treated, irradiated cells of S mutant or WT BCRF1 recombinant-infected LCLs were similar (Table 1).

**EBV recombinants with BCRF1 completely deleted are also**

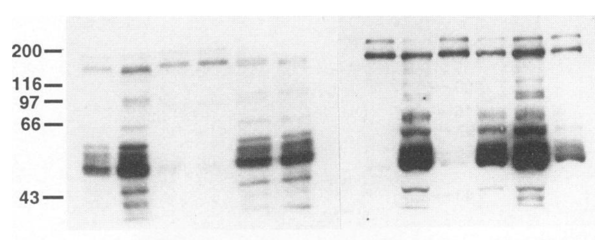


FIG. 7. Levels of induced virus replication were compared by immunoblotting lysates from TPA-treated LCLs (indicated by the suffix "T") with human sera reactive against lytic infection-associated viral proteins. WT BCRF1-infected cell lines 8 and 35 and S mutant BCRF1-infected cell lines 20, 36, and 41 were treated with TPA or medium for 72 h. Molecular sizes are shown at the left in kilodaltons.

TABLE 1. Transformation by recombinant virus[a]

| LCL | No. of transformants/$10^4$ cells |
|---|---|
| BCRF1 negative | |
| 36 | 1.49 |
| 41 | 0.48 |
| D | 1.20 |
| N | 1.20 |
| HH | 1.16 |
| OO | 0.96 |
| BCRF1 positive | |
| 8 | 1.73 |
| H | 1.48 |
| K | 1.00 |
| J | 0.88 |
| B | 1.28 |

[a] Dilutions of aliquots of each LCL were TPA treated to induce EBV replication, irradiated, and cocultivated with PBMCs in 96-well plates. Four to six weeks after plating, the number of newly transformed cell lines was counted. Results are expressed as the number of transformants obtained per $10^4$ cells of each LCL used as the source of infecting virus.

**not different from WT in initiation of latent infection, in growth transformation, and in lytic virus infection in vitro.** A 3,335-bp deletion was made in the S mutant BCRF1 SalA cosmid by cleavage with *Xba*I and BAL 31 digestion. The deletion was sequenced, revealing removal of bp 9535 to 12870, including all of the BCRF1 ORF (3). The BCRF1-deleted cosmid was transfected into P3HR-1 cells along with pSVNaeZ to induce lytic EBV replication. The resulting progeny virus was used to infect primary B lymphocytes as in the derivation of the S mutant and WT BCRF1 recombinants. The resultant transformation marker-rescued LCLs were screened by PCR using the previously described oligonucleotides, which results in the amplification of a 231-bp fragment from WT BCRF1 and no fragment from the deleted genome. Of 44 transformation marker rescued LCLs, 27 (61%) contained WT BCRF1 DNA. Seventeen (39%) had only the deleted BCRF1 DNA. In reconstruction experiments, 1 in 1,000 WT BCRF1 genomes would have been detected in PCR experiments with primers specific for WT BCRF1 (data not shown). The LCLs infected only with a BCRF1-deleted EBV recombinant grew indistinguishably from WT BCRF1 recombinant-infected LCLs derived in parallel. Replication of the BCRF1-deleted EBV recombinants could be induced with TPA, and the virus could be passaged to transform primary B lymphocytes by irradiation and cocultivation. Eight of the BCRF1-deleted virus mutants were passaged in this manner. Transformed cell lines became macroscopically visible 3 to 4 weeks after cocultivation and were expanded to growth in bulk without difficulty, as is characteristic of WT recombinant virus passaged by cocultivation (38). Growth of the newly transformed LCLs at 6 weeks was also indistinguishable from growth of LCLs infected with WT BCRF1 recombinants, with a doubling time of 48 to 72 h. This assay can detect differences in growth-transforming properties of mutant EBV recombinants as shown for mutant EBNA-LP EBV recombinants (23). These data confirm that BCRF1 is not necessary for initiation of primary B-lymphocyte growth transformation and is not necessary for EBV replication in LCLs. Since this extensive deletion removes elements involved in regulation of EBNA expression from the EBV genome (4, 32, 44), the molecular biology of these recombinants is being intensively investigated.

## DISCUSSION

These experiments were undertaken to evaluate the role of BCRF1 in EBV infection and to specifically evaluate the possibility that BCRF1 is important to B-lymphocyte growth transformation or in modifying the host response to virus infection. Most experiments compared WT BCRF1 EBV recombinants with S mutant BCRF1 EBV recombinants. The S mutant BCRF1 is truncated by the insertion of stop codons in all three reading frames after BCRF1 codon 116. The mutation is predicted to result in the translation of a 116-residue BCRF1 cross-reactive protein which would be 91 amino acids after cleavage of the predicted cleavable signal peptide. There are no data on the biologic activity of truncated IL-10 or BCRF1 proteins. However, the extensive conservation of primary sequence between human IL-10, BCRF1, and even murine IL-10, especially at the carboxy-terminal half of the protein (17), is consistent with the hypothesis that the last 54 amino acids are important for normal BCRF1 function. An experimental test of this hypothesis in the experiments reported here confirmed a significant difference from WT BCRF1 in IFN-$\gamma$-inhibiting activity. Since the possibility could not be excluded that the amino-terminal 91-amino-acid cross-reactive protein retained some BCRF1 activity, we constructed a second group of mutated EBV recombinants from which the entire BCRF1 gene was deleted. Both types of mutants did not differ from WT BCRF1 recombinants in the ability to initiate or maintain latent B-lymphocyte infection or growth transformation, and both were able to undergo lytic infection. Thus, these experiments exclude an essential or critical role for BCRF1 at the cellular level in initiating or maintaining latent B-lymphocyte infection or growth transformation. This is an important point, since recombinant BCRF1 and human IL-10 can stimulate DNA replication and immunoglobulin synthesis in purified tonsillar B lymphocytes which are coactivated by surface CD40 ligation (33). BCRF1 secreted from lytically infected cells could have had a beneficial effect on initiation of latent infection or on the initiation of cell transformation. No such effect was evident in comparative passaging of mutated and WT BCRF1 EBV recombinants when the lytically infected cells were cocultivated with primary B lymphocytes.

A late lytic infection protein such as BCRF1 could also foster the growth of cells in tissues in vivo if there is a significant fraction of cells permissive for EBV replication in the tissue. Expression of BCRF1 has been suggested in AIDS-associated EBV-positive lymphomas (7), and expression of other late lytic genes has been detected in SCID mouse tissues (34). With the caveat that the S mutant BCRF1 could have some residual BCRF1 activity, no difference was evident in tumorigenicity or lethality of cells infected with S mutant or WT BCRF1 recombinants.

In contrast to the lack of effects on B lymphocyte growth or tumorigenicity, significant effects of WT BCRF1 in blocking IFN release were readily demonstrated, highlighting the importance of this aspect of IL-10 activity in BCRF1 function. Recombinant IL-10 or BCRF1 can inhibit IFN-$\gamma$ synthesis by human PBMCs stimulated by phytohemagglutinin or anti-CD3 (42). In addition to inhibition of T-lymphocyte IFN-$\gamma$ secretion, IL-10 and BCRF1 can inhibit IL-2-induced IFN-$\gamma$ secretion by the NK cell component of human PBMCs (14). IFN-$\gamma$ was one of the first cytokines shown to be important in modulating the outcome of EBV infection. PBMCs from seronegative donors can inhibit but do not abolish B-lymphocyte proliferation and immunoglobulin synthesis in response to EBV infection (40). This nonimmune delay of transformation is believed to be mediated primarily by IFN-$\gamma$ secretion. The source of IFN-γ in these EBV-infected PBMC cultures from seronegative donors was not determined. However, human NK cells (45), as well as $CD4^+$ T cells from neonates (1), can produce IFN-γ and may be important in the nonimmune cytokine response to EBV infection. Thus, despite its designation as immune IFN, IFN-γ may be important in controlling EBV infection in both nonimmune and immune humans. Consistent with such a role is the finding of elevated IFN-γ levels in the serum of individuals with acute infectious mononucleosis (19). Our experiments also utilized LCLs and PBMCs from an adult seronegative donor, suggesting that the observed IFN-γ response is due to nonspecific immunity and may be NK cell mediated. The levels of IFN-γ detectable in these cultures are similar to those which can block primary B-lymphocyte transformation in vitro (20, 38). IFN-γ secretion also occurs in PBMCs from seropositive donors infected with cell-free virus; in this case, the primary source of secreted IFN is believed to be the $CD4^+$ T lymphocyte (2). The WT BCRF1 effect in inhibiting IFN-γ secretion, which is delineated by comparison with the S mutant BCRF1, is therefore likely to be important in EBV infection, in vivo, as part of a block to immune and nonimmune host responses, including a direct antiviral effect of the IFN-γ.

In addition to IFN induction, primary EBV infection in vivo results in the rapid appearance of EBV-specific and nonspecific cytotoxicity (30). After primary EBV infection, $CD8^+$ cytotoxic T lymphocytes specific for EBNAs (except EBNA1) and LMPs are maintained for life (31, 27). The importance of cellular immunity in controlling primary and persistent EBV infection is underscored by the fulminant lymphoproliferative syndromes which occur in individuals with acquired or inborn defects in cell-mediated immunity. Similarly, in vitro regression of transformed B-lymphocyte outgrowth from seropositive PBMCs is mediated by EBV-specific $CD8^+$ cytotoxic T lymphocytes (30). Experiments examining the role of viral IL-10 in infection, using a recombinant vaccinia virus expressing IL-10 as a model, found a lower NK and $CD8^+$ cytotoxic response to the IL-10 expressed by vaccinia virus in mice (18). Thus BCRF1 secreted from EBV-infected cells could also play a role in down regulating cell-mediated responses to primary and persistent infection. However, under some circumstances, IL-10 has a paradoxical effect on cytotoxic responses. Addition of IL-10 made by transfected EBV-negative Burkitt lymphoma cells to PBMCs from seropositive human donors enhanced the generation of EBV-specific and nonspecific cytotoxicity (37). Further, in SCID mice, vaccinia virus expressing murine IL-10 enhanced generation of NK activity (18). The net effect of BCRF1 in modulating the host immune response may therefore vary according to the specific in vivo situation and the concentrations of BCRF1 involved. For example, during initial infection, BCRF1 may enable the virus to establish latent infection and may be necessary to permit subsequent reactivation. In the immune host, BCRF1 secretion may actually serve to enhance the cytotoxic response to virus reactivation in certain tissues and allow the virus to maintain its benign persistent state. Maintenance of a nonpathogenic infection which nevertheless allows intermittent EBV replication in oropharyngeal epithelium is centrally important for EBV to maintain itself in human populations.

The WT BCRF1 effect on IFN secretion that we have described, the data on BCRF1 effects on cell-mediated cytotoxicity, and the observation that recombinant BCRF1 lacks the thymocyte-stimulating activity of recombinant IL-10 (22) are compatible with the hypothesis that EBV has captured a human cytokine gene and retained those activities necessary to down modulate specific and nonspecific host responses in order to establish and maintain long-term latency in B lymphocytes. The assay for BCRF1 effect on inhibiting IFN-γ production in mixed LCL-PBMC cultures is the first functional assay for WT BCRF1 secreted in lytic EBV infection. The sensitivity of the assay is consistent with biologic relevance. The cellular elements essential for the effects shown here and the role of immunity in the assay response require further delineation.

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## REFERENCES

1. **Andersson, U., S. Britton, M. DeLey, and G. Bird.** 1983. Evidence for the ontogenic precedence of suppressor T cell functions in the human neonate. Eur. J. Immunol. **13:**6–13.
2. **Andersson, U., O. Martinez-Maza, J. Andersson, S. Britton, H. Gadler, M. DeLey, and S. Modrow.** 1984. Secretion of gamma interferon at the cellular level. Induction by Epstein-Barr virus. Scand. J. Immunol. **20:**425–432.
3. **Baer, R., A. T. Bankier, and M. D. Biggin.** 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. Nature (London) **310:**207–211.
4. **Bodescot, M., M. Perricaudet, and P. J. Farrell.** 1987. A promoter for the highly spliced family of RNAs of Epstein-Barr virus. J. Virol. **61:**3424–3430.
5. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation acid guanidium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
6. **Cohen, J., F. Wang, and E. Kieff.** 1989. Epstein-Barr virus nuclear protein 2 is a key determinant of lymphocyte transformation. Proc. Natl. Acad. Sci. USA **86:**9558–9562.
7. **Emilie, D., R. Touitou, M. Raphael, M. Peuchmau, O. Devergnee, D. Rea, J. Coumbraras, M.-C. Crevon, L. Edelman, I. Joab, and P. Galanaud.** 1992. In vivo production of interleukin-10 by malignant cells in AIDS lymphomas. Eur. J. Immunol. **22:**2937–2942.
8. **Fiorentino, D., M. Bond, and T. Mosmann.** 1989. Two types of murine helper T cell clone. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. J. Exp. Med. **170:**2081–2095.
9. **Fiorentino, D., A. Zlotnik, P. Vieira, T. Mossmann, M. Howard, K. Moore, and A. O'Garra.** 1991. IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. J. Immunol. **146:**3444–3451.
10. **Go, N., B. Castle, R. Barrett, R. Kastelein, W. Dang, T. Mossmann, K. Moore, and M. Howard.** 1990. Interleukin 10, a novel B cell stimulatory factor: unresponsiveness of X chromosome-linked immunodeficiency B cells. J. Exp. Med. **172:**1625–1631.
11. **Hammerschmidt, W., and B. Sugden.** 1989. Genetic analysis of immortalizing functions of Epstein-Barr in human B lymphocytes. Nature (London) **340:**393–397.
12. **Henle, G., and W. Henle.** 1979. The virus as the etiologic agent of infectious mononucleosis, p. 61–78. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein-Barr virus. Springer-Verlag, Berlin.
13. **Hsu, D., R. De Waal Malefyt, D. Fiorentino, M. Dang, P. Vieira, J. Devries, H. Spits, T. Mosmann, and K. Moore.** 1990. Expression of interleukin-10 activity by Epstein-Barr virus protein BCRF1. Science **250:**830–832.
14. **Hsu, D., and K. Moore.** 1992. Differential effects of IL-4 and IL-10 on IL-2-induced and IFN-gamma synthesis and lymphokine-activated killer activity. Int. Immunol. **4:**563–569.
15. **Hudson, G., A. Bankier, S. Satchwell, and B. Barrell.** 1985. The short unique region of the B95-8 Epstein-Barr virus genome. Virology **147:**81–98.
16. **Kieff, E., F. Wang, M. Birkenbach, J. Cohen, J. Sample, B.**

**Tomkinson, S. Swaminathan, R. Longnecker, A. Marchini, J. Mannick, S. Tsang, C. Sample, and M. Kurilla.** 1991. Molecular biology of lymphocyte transformation by Epstein-Barr virus, p. 563–576. *In* J. Brugge et al. (ed.), Origins of human cancer: a comprehensive review. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

17. **Kim, J., C. Brannan, N. Copeland, N. Jenkins, T. Khan, and K. Moore.** 1992. Structure of the mouse interleukin-10 gene and chromosomal localization of the mouse and human genes. J. Immunol. **148:**3618–3623.
18. **Kurilla, M., S. Swaminathan, R. Welsh, E. Kieff, and R. Brutkiewicz.** 1993. Effects of virally expressed interleukin-10 on vaccinia virus infection in mice. J. Virol. **67:**7623–7628.
19. **Linde, A., B. Andersson, S. B. Svenson, H. Ahrne, M. Carlsson, P. Forsberg, H. Hugo, A. Karstorp, R. Lenkei, A. Lindwall, A. Loftenius, C. Sall, and J. Andersson.** 1992. Serum levels of lymphokines and soluble cellular receptors in primary Epstein-Barr virus infection and in patients with chronic fatigue syndrome. J. Infect. Dis. **165:**994–1000.
20. **Lotz, M., C. Tsoukas, S. Fong, D. Carson, and J. Vaughan.** 1985. Regulation of Epstein-Barr virus infection by recombinant interferons. Selected sensitivity to interferon-gamma. Eur. J. Immunol. **15:**520–525.
21. **Lotz, M., C. Tsoukas, S. Fong, C. Dinarello, D. Carson, and J. Vaughan.** 1986. Release of lymphokines after Epstein-Barr virus infection in vitro. I. Sources of and kinetics of production of interferons and interleukins in normal humans. J. Immunol. **136:**3636.
22. **MacNeil, I., T. Suda, K. Moore, T. Mossmann, and A. Zlotnik.** 1990. IL-10, a novel growth cofactor for mature and immature T cells. J. Immunol. **145:**4167–4173.
23. **Mannick, J., J. I. Cohen, M. Birkenbach, A. Marchini, and E. Kieff.** 1991. The Epstein-Barr virus nuclear protein encoded by the leader of the EBNA RNAs is important in B-lymphocyte transformation. J. Virol. **65:**6826–6837.
24. **Miller, G.** 1990. Epstein-Barr virus; biology, pathogenesis and medical aspects, p. 1921–1951. *In* B. Fields and D. Knipe (ed.), Virology. Raven Press, New York.
25. **Moore, K., P. Vieira, D. Fiorentino, M. Trounstine, T. Khan, and T. Mosmann.** 1990. Homology of cytokine synthesis inhibitory factor (IL-10) to the Epstein-Barr virus gene BCRFI. Science **248:**1230–1234.
26. **Mosier, D., R. Gulizia, S. Baird, and D. Wilson.** 1988. Transfer of a functional human immune system to mice with severe combined immunodeficiency. Nature (London) **335:**256–259.
27. **Moss, D. J., A. B. Rickinson, and J. H. Pope.** 1978. Long term T cell mediated immunity to Epstein-Barr virus in man. I. Complete regression of virus-induced transformation in cultures of seropositive donor leukocytes. Int. J. Cancer **23:**618–625.
28. **Mossman, T., H. Cherwinski, M. Bond, M. Giedlin, and R. Coffman.** 1986. Two types of murine helper T cell clone. J. Immunol. **136:**2348–2357.
29. **Rabson, M., L. Gradoville, L. Heston, and G. Miller.** 1982. Nonimmortalizing P3J-HR1 Epstein Barr virus: a deletion mutant of its transforming parent, Jijoye. J. Virol. **48:**834–844.
30. **Rickinson, A. B.** 1986. Cellular immunological responses to the virus infection, p. 75–125. *In* M. A. Epstein and B. G. Achong (ed.), The Epstein Barr virus: recent advances. William Heinemann Medical Books, London.
31. **Rickinson, A. B., D. J. Moss, and L. E. Wallace.** 1981. Long term T cell-mediated immunity to Epstein-Barr virus. Cancer Res. (Suppl.) **41:**4216–4221.
32. **Rooney, C., M. Brimmell, M. Buschle, G. Allan, P. Farrell, and J. Kolman.** 1992. Host cell and EBNA-2 regulation of Epstein-Barr virus latent cycle promoter activity in B lymphocytes. J. Virol. **66:**496–504.
33. **Rousset, F., E. Garcia, T. DeFrance, C. Peronne, N. Vezzio, D. Hsu, R. Kastelein, K. Moore, and J. Banchereau.** 1992. Interleukin 10 is a potent growth and differentiation factor for activated human B lymphocytes. Proc. Natl. Acad. Sci. USA **89:**1890–1893.
34. **Rowe, M., L. Young, J. Crocker, H. Stokes, S. Henderson, and A. Rickinson.** 1991. EBV-associated lymphoproliferative disease in the SCID mouse model: implications for the pathogenesis of EBV-positive lymphomas in man. J. Exp. Med. **173:**147–158.
35. **Skare, J., J. Farley, J. L. Strominger, O. Fresen, M. S. Cho, and H. zur Hausen.** 1985. Transformation by Epstein-Barr virus requires DNA sequences in the region of *Bam*HI fragments Y and H. J. Virol. **55:**286–297.
36. **Spits, H., and R. de Waal Malefyt.** 1992. Functional characterization of human IL-10. Int. Arch. Allergy Immunol. **99:**8–15.
37. **Stewart, J. P., and C. M. Rooney.** 1992. The interleukin-10 homolog encoded by Epstein-Barr virus enhances the reactivation of virus-specific cytotoxic T cell and HLA-unrestricted killer cell responses. Virology **191:**773–782.
38. **Swaminathan, S., B. Huneycutt, C. Reiss, and E. Kieff.** 1992. Epstein-Barr virus small RNAs (EBERs) do not modulate interferon in infected B cells. J. Virol. **66:**5133–5136.
39. **Swaminathan, S., B. Tomkinson, and E. Kieff.** 1991. Recombinant Epstein-Barr virus with small RNA (EBER) genes deleted transforms lymphocytes and replicates in vitro. Proc. Natl. Acad. Sci. USA **88:**1546–1550.
40. **Thorley-Lawson, D. A.** 1981. The transformation of adult but not newborn human lymphocytes by Epstein Barr virus and phytohemagglutinin is inhibited by interferon: the early suppression by T cells of Epstein Barr infection is mediated by interferon. J. Immunol. **126:**829–833.
41. **Tomkinson, B. E., D. K. Wagner, D. L. Nelson, and J. L. Sullivan.** 1987. Activated lymphocytes during acute Epstein-Barr virus infection. J. Immunol. **139:**3802–3807.
42. **Vieira, P., R. de Waal-Malefyt, M. Dang, K. Johnson, R. Kastelein, D. Fiorentino, J. deVries, M. Roncarolo, T. Mosmann, and K. Moore.** 1991. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1. Proc. Natl. Acad. Sci. USA **88:**1172–1176.
43. **Wang, F., C. D. Gregory, M. Rowe, A. B. Rickinson, D. Wang, M. Birkenbach, H. Kikutani, T. Kishimoto, and E. Kieff.** 1986. Epstein-Barr virus nuclear antigen 2 specifically induces expression of the B cell activation antigen CD23. Proc. Natl. Acad. Sci. USA **84:**3452–3456.
44. **Woisetschlaeger, M., J. Strominger, and S. Speck.** 1989. Mutually exclusive use of viral promoters in Epstein-Barr virus latently infected lymphocytes. Proc. Natl. Acad. Sci. USA **86:**6498–6502.
45. **Young, H. A., and J. R. Ortaldo.** 1987. One-signal requirement for interferon-gamma production by human large granular lymphocytes. J. Immunol. **139:**724–727.